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(71) Applicant

Bio-Rad Laboratories Inc

(Incorporated in the USA – Delaware)

1000 Alfred Nobel Drive, Hercules, California 94547,
United States of America

(72) Inventors

George Bers

Franklin R. Witney

(74) Agent and/or Address for Service

Carpmaels & Ransford

43 Bloomsbury Square, London, WC1A 2RA,
United Kingdom

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(54) Detection and imaging in biochemical assays using phosphorescent screens

(57) Immobilized macromolecules, such as proteins or nucleic acid sequences (12), are tagged with labels (13) which induce a chemiluminescent reaction in a liquid-phase substrate (15). As the reaction is occurring, the substrate is exposed to a phosphorescent screen (16) which absorbs the chemiluminescent emission in an image of the pattern formed by the macromolecules. The absorbed energy is then released and read, providing qualitative and optionally quantitative information regarding the immobilized macromolecules. Distinct labels, which react with a mixture of chemiluminescent substrates to emit light at correspondingly distinct wavelengths, may be used with a phosphorescent screen selectively excitable by light of said distinct wavelengths. By this method different macromolecules can be detected simultaneously.

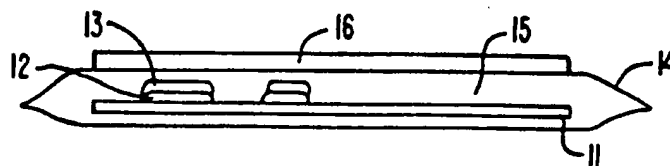


FIG. 1.

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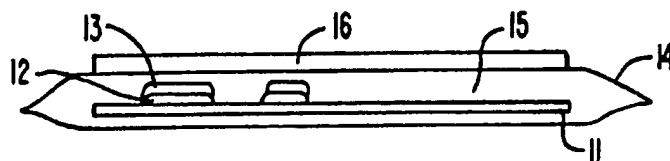


FIG. 1.

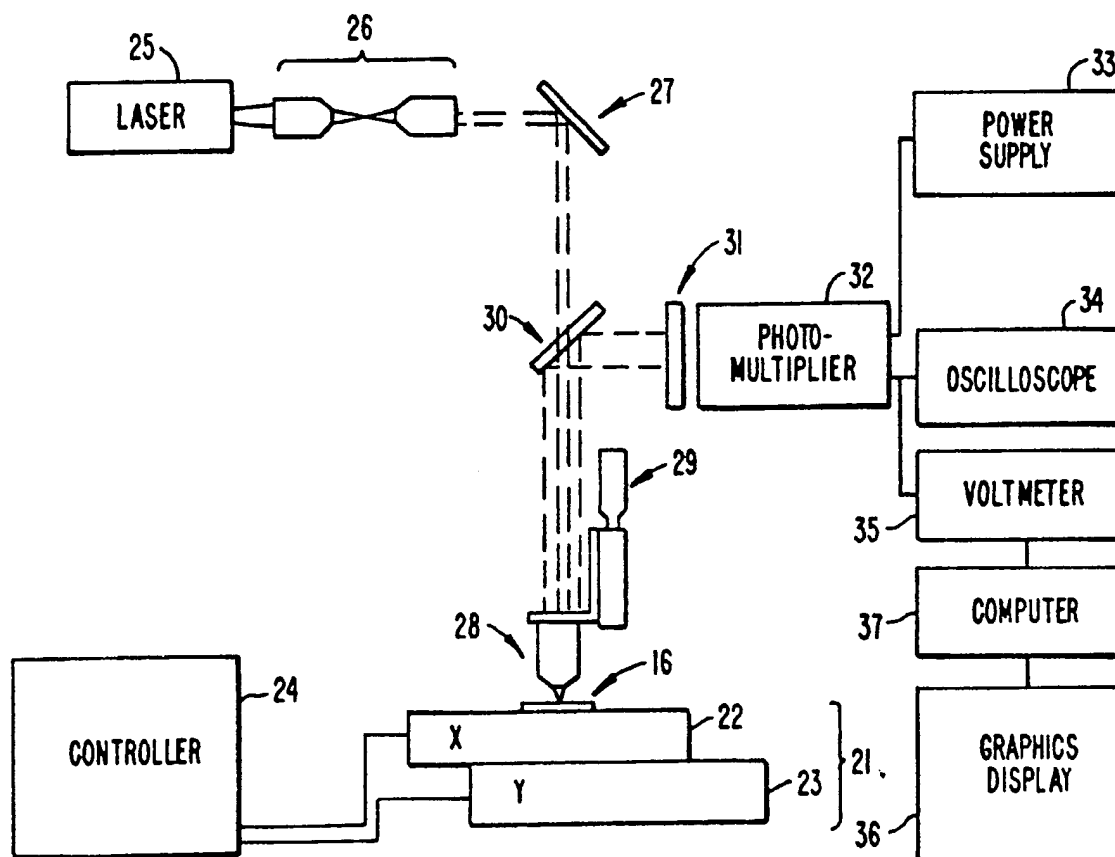


FIG. 2.

DETECTION AND IMAGING IN BIOCHEMICAL ASSAYS
USING PHOSPHOR SCREENS

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This invention lies in the general field of biochemical assays and detection methods, with a focus on methods of labelling species and detecting the labels. In particular, the invention relates to macromolecule detection involving nonisotopic labelling methods.

A procedure essential to all molecular biology laboratories is the detection and imaging of macromolecules. This is used in protein assays, DNA sequencing, gene mapping, and any number of other experiments and determinations. The most common method used is by tagging or labelling the molecule or sequence of interest with a radioactive species, then recording an autoradiographic image of the radioactive emission on x-ray film.

Disadvantages associated with the use of radioactive species are that they present a handling hazard to the laboratory technician, they are difficult to dispose of, and their radioactive decay requires that they be periodically replaced with fresh materials. Labels which do not involve radioactive species are not readily translated into spatial images on a film or screen, and are generally of lower sensitivity than their radioactive counterparts.

X-ray films also have their limitations. The dynamic range of a typical x-ray film is about fiftyfold, which limits the degree to which one can obtain quantitative information from the film. Also, long exposures are generally required to obtain a satisfactory image, due to the limited sensitivity of the film to the β -particle emissions used in most radioactive labels. In addition, variability is potentially introduced in the development of the film, since this requires a number of steps involving unstable solutions.

A method has now been developed for using phosphor
screens in combination with chemiluminescence to detect and
5 image macromolecules, replacing the need for radioisotopes.
The macromolecules may now be selectively labelled with a
species which induces a chemiluminescent reaction in a
substrate, and the substrate exposed to a phosphor screen,
which is capable of responding to the chemiluminescent emission
10 and indicating its occurrence in a readily detectable manner.

The use of the substrate as an intermediate element
between the label and the film or screen permits one to control
the formation of the image and the intensity of the signal and
thus the sensitivity of the experiment, while avoiding the
15 hazard of radioactive materials. Furthermore, the substrate is
in liquid form, which provides further advantages in terms of
enhancing its contact with the label, and in offering further
parameters for use in controlling and forming the image, such
parameters including concentration, light transmissivity and
20 the ability to accomodate variations in the physical
arrangement and configuration of the solid elements of the
system.

The use of the phosphor screen provides an image with
a high degree of sensitivity, contrast and reproducibility with
25 relatively short exposure times. Further advantages include
the fact that phosphor screens do not require chemical
treatment to be readable, and that they can trap a
chemiluminescent emission upon receiving it, and retain the
energy of the emission until stimulated by an external source
30 such as infrared light whereupon the screen releases its own
corresponding emission.

Other features, advantages and preferred embodiments
of the invention will be apparent from the description which
follows, making particular reference to the drawings, in which:-

35

FIG. 1 is a representation of an experiment conducted in accordance with the present invention, in which a macromolecular immobilization pattern is transferred to a phosphor screen.

FIG. 2 is a representation of an optical system for generating and detecting signals which translate the pattern on the phosphor screen to a visually readable form.

10

Basic elements of the invention are the label, the chemiluminescent substrate and the phosphor screen. This detailed discussion will begin with a description of the label and chemiluminescent substrate.

The label and the substrate may be any materials tending to produce a chemiluminescent emission upon contact, including the wide variety of species known in the chemiluminescence art. The label and the substrate may, for example, be reactants which combine to form an excited state which spontaneously degenerates to the ground state with the release of a fluorescent or phosphorescent emission, or reactants which combine to form an intermediate which decomposes spontaneously to an excited state which then undergoes the same conversion and energy release. Alternatively, the reaction may be entirely contained in the substrate. The substrate in such a reaction may be a single species and the reaction either a conversion or decomposition entailing an emission, or the substrate may be a mixture of species which enter into a reaction which results in the emission.

For substrate-contained reactions, the label may be a catalyst or an enzyme. Catalyst and enzyme labels are preferred, particularly enzyme labels, due to their successive and continued interaction with the reactant substrate molecules. This permits emissions to continue for prolonged periods, thereby facilitating recordation and detection, and

provides signal amplification which can be controlled by the amount of substrate made available to the label.

5 A wide variety of catalysts and enzymes capable of inducing a chemiluminescent reaction are known, and therefore suitable for use in the practice of the present invention. Examples of catalysts are various metals and such species as adenosine triphosphate. Examples of enzymes are lactate dehydrogenase, luciferase and phosphatases such as alkaline phosphatase (AP). Enzymes are preferred, with phosphatases
10 being particularly preferred, and alkaline phosphatase the most preferred.

Any known chemiluminescent reaction may be utilized in the present invention. Peroxide decomposition reactions are a convenient class. Preferred reactions within this class are
15 1,2-dioxetane decompositions, including decompositions of 1,2-dioxetanones (α -peroxylactones) and 1,2-dioxetanediones (peroxyoxalates). Examples of other classes are reactions involving luminol (3-aminophthalhydrazide) and its analogs, and organometallics such a *p*-chlorophenylmagnesium bromide.

20 Particularly preferred reactions are 1,2-dioxetane decompositions regulated by enzymes. Compounds containing the 1,2-dioxetane group as well as an ester group of an orthophosphoric acid are an example of one class of compounds meeting this description. Decomposition of these compounds is
25 catalyzed by a phosphatase. One particular example is the substrate 3-(2'-spiroadamantane)-4-methoxy-4-(3"-phosphoryloxy)phenyl-1,2-dioxetane with the enzyme alkaline phosphatase. The former is commercially available from Tropix Corporation, Bedford, Massachusetts, and from Lumigen
30 Corporation, Detroit, Michigan, and the latter is commercially available from a wide range of sources.

The substrate will be in the liquid phase. Accordingly, the most convenient substrates will be water-soluble substances, and the liquid phase will be an aqueous
35 solution. It is understood that the amount of substrate which will undergo the reaction may not be readily determinable, since the presence and amount of macromolecular species, and hence attached label, may not be known. Nevertheless, one

would seek to include an excess of substrate, based on either the expected amount or upper limit of macromolecular species being detected, so that all label immobilized by attachment to the macromolecular species enters into the reaction and the level of emission can be related to the amount of label. With catalyst or enzyme labels, a large excess of substrate is preferred, such that chemiluminescent light emissions will continue for sufficient time to allow the remaining steps of the procedure to be carried out while light of a sufficient intensity continues to be emitted. In particularly preferred systems, sufficient substrate is included to result in chemiluminescent light emissions continuing for at least about one hour, most preferably at least about 24 hours.

Turning next to the phosphor screen, a wide variety of phosphors may be used. The proper selection in each particular application will depend on the chemiluminescent material, the phosphors being selected to receive and respond to the emission produced by the particular chemiluminescent material.

Phosphors used in the practice of the invention may be selected from the full range of materials known to possess the capability of phosphorescence. In general, these are materials which absorb light and enter an excited state as a result, then undergo relaxation to the ground state while emitting light, either at a different intensity or frequency or over a different time scale, or both. Materials meeting this description include natural minerals, biological compounds and synthetically prepared materials and blends. Examples are metal halophosphates such as $\text{Ca}_5(\text{PO}_4)_3(\text{F}, \text{Cl}):\text{Sb}(\text{III}), \text{Mn}(\text{II}), \text{Sr}_5(\text{PO}_4)_3(\text{Cl}):\text{Eu}(\text{II}), \text{Sr}_5(\text{PO}_4)_3(\text{F}, \text{Cl}):\text{Sb}(\text{III}), \text{Mn}(\text{II})$ and $[\text{SrEu}(\text{II})]_5(\text{PO}_4)_3\text{Cl}$; other rare-earth-activated phosphors such as $\text{Y}_2\text{O}_3:\text{Eu}(\text{III}), \text{SrB}_4\text{O}_7:\text{Eu}(\text{II}), \text{BaMg}_2\text{Al}_{16}\text{O}_{27}:\text{Eu}(\text{II}), \text{Y}(\text{VO}_4):\text{Eu}(\text{III}), \text{Y}(\text{VO}_4)\text{PO}_4:\text{Eu}(\text{III}), \text{Sr}_2\text{P}_2\text{O}_7:\text{Eu}(\text{II}), \text{SrMgP}_2\text{O}_7:\text{Eu}(\text{II}), \text{Sr}_3(\text{PO}_4)_2:\text{Eu}(\text{II}), \text{Sr}_5\text{Si}_4\text{Cl}_6\text{O}_{10}:\text{Eu}(\text{II}), \text{Ba}_2\text{MgSi}_2\text{O}_7:\text{Eu}(\text{II}), \text{GdOS}:\text{Tb}(\text{III}), \text{LaOS}:\text{Tb}(\text{III}), \text{LaOBr}:\text{Tb}(\text{III}), \text{LaOBr}:\text{Tm}(\text{III})$ and $\text{Ba}(\text{F}, \text{Cl})_2:\text{Eu}(\text{II})$; other aluminate-host phosphors such as $\text{Ce}_{0.65}\text{Tb}_{0.35}\text{MgAl}_{11}\text{O}_{19}$; silicate-host phosphors such as $\text{Zn}_2\text{SiO}_4:\text{Mn}(\text{II})$; and fluoride-host phosphors

such as $\text{Y}_{0.79}\text{Yb}_{0.20}\text{Er}_{0.01}\text{F}_3$, $\text{La}_{0.86}\text{Yb}_{0.12}\text{Er}_{0.02}\text{F}_3$, and $\text{Y}_{0.639}\text{Yb}_{0.35}\text{Tm}_{0.001}\text{F}_3$.

Of particular interest are phosphors which remain in the excited state until released by external stimulation.

- 5 These include many of those listed above, plus others. Preferred examples are alkaline earth metal sulfides and selenides, doped with samarium and europium or cerium oxide, sulfide or fluoride, and further containing a fusable salt such as lithium fluoride, barium sulfate or both serving as a flux.
- 10 Lists and descriptions of such materials are found in United States Patent Nos. 4,812,660 (March 14, 1989), 4,822,520 (April 18, 1989) and 4,830,875 (May 16, 1989), to Lindmayer, J. (Quantex Corporation), each of which is incorporated herein by reference. The stimulation which releases the energy may be in
- 15 the form of heat or electromagnetic radiation, such as visible light, x-rays, ultraviolet radiation and infrared radiation, depending on the type of phosphor.

- The substrate and phosphors will be selected to emit and absorb, respectively, at the same wavelength, thereby
- 20 complementing each other in terms of energy emission and response. The energy of a single emission will generally be in the form of a wavelength band, the width of which is not critical in the general sense. In certain applications, as described in more detail below, narrowly defined band widths
- 25 will serve specific functions. As for the actual wavelengths of the emissions, in most applications within the contemplation of this invention, emissions with peak wavelengths falling within the range of about 350nm to about 700nm, preferably from about 400nm to about 600nm will be used.

- 30 The use of mixed phosphors, together with a corresponding mixture of label/substrate systems, presents further opportunities for enhanced use of the invention. For example, luminol, a common currently available chemiluminescent substrate, emits light at 428nm when activated, and various
- 35 naphthyl dioxetane isomers currently available emit light at wavelengths varying from 463nm to 560nm. Of the various phosphors available from Quantex Corporation, the phosphor designated Q-16 will respond to wavelengths of 470nm but not

higher, while the phosphor designated Q-42 will respond to wavelengths up to about 600nm.

With these phosphors combined on a single screen, or any other combination which can similarly discriminate, one can use multiple label/substrate systems of wavelengths corresponding to those to which the phosphors are receptive. The labels may be selectively placed on distinct preselected groups of macromolecules, and the substrates may be combined in a single substrate mixture.

Since the phosphors will themselves emit light at distinct wavelengths, discrimination in the read-out process may be achieved in a variety of ways, depending on the particular read-out process used. Using infrared detection for read-out, for example, a photomultiplier tube can discriminate between the Q-16 light emissions, which are green, and the Q-42 light emissions, which are orange, through the use of filters.

As a further example of combined systems utilizing the present invention, mixed phosphors may be used with both chemiluminescent and radioactive emissions in a multi-signal system. For example, one set of nucleic acid molecules (or other macromolecules) may be tagged with a label such as alkaline phosphatase which induces a chemiluminescent reaction in the substrate, such as one which emits light at 600nm, and another set tagged with phosphorus-32, or some other radioisotope, which emits β radioactive emissions. A mixed phosphor screen such as a Q-16 and Q-42 combination may then be used. Since the Q-42 phosphor is insensitive to the β radioactive emissions, those macromolecules tagged with phosphorus-32 would be imaged only by the Q-16. The Q-42 phosphor would however be sensitive to the 600nm emissions from the chemiluminescent substrate. A photomultiplier tube could discriminate between the light emissions from the two phosphors in the manner described in the preceding paragraph.

The use of mixed phosphors in these and other combination systems permits an unlimited variety of comparisons and discriminations. For example, one can discriminate between distinct groups of macromolecules in a single sample or compare

against an internal standard. Other possibilities will be readily apparent to those skilled in the art.

The method of the invention may serve as either a detection method, a quantitation method or both. It may be utilized in assays or other determinations in a variety of configurations and arrangements, which will be readily apparent to those skilled in the art. In general, the invention is applicable to any procedure for detecting or quantifying an immobilized macromolecule or portion of a macromolecule. It is of particular interest in imaging spatial arrays of macromolecules, since it can be conducted in a manner which provides localized information. Such imaging is of value for spatial arrays generated by a variety of laboratory procedures, including electropherograms, chromatograms, dot blots, and any other arrangement of separated solutes or species.

The term "immobilized" is used in this specification to denote retention of a species in a fixed location on a non-liquid surface or matrix, in a manner by which the species will not become dislodged or unattached upon contact with the liquid-phase chemiluminescent substrate. The non-liquid surface or matrix may be a slab gel such as a polyacrylamide or agarose gel, a blotting membrane such as nitrocellulose or derivatized nylon, or a solid surface such as coated glass or a plastic microtiter plate.

Selective tagging of the macromolecule of interest with the label may be achieved by any of the various means known in the biochemical art. The attachment may be a covalent bond, an affinity-type bond, a hydrophobic interaction, a hybridization-type interaction or any other means of attachment. Selectivity may be inherent in the means of attachment, as in covalent, hydrophobic and hybridization interactions, or it may be the result of immunological-type binding specificity or other specific binding behavior. Preferred interactions are hybridization interactions such as the use of DNA or RNA probes, and specific binding interactions, such as antigen-antibody interactions and avidin-biotin interactions.

In these preferred interactions, the label is conjugated to an appropriate binding member which binds to the immobilized macromolecule. The latter is thus "tagged" with the label selectively, i.e., to the exclusion of the surface or
5 matrix itself and of the other macromolecules which lack the specific binding characteristics involved in the attraction. The conjugate is formed in the conventional manner through a covalent bond, including in some cases a cross-linker when one is desired or beneficial.

10 The method of the present invention is performed by immersing the solid phase on which the macromolecules of interest are immobilized in the liquid-phase substrate, the macromolecules being tagged with the labels described above so that the chemiluminescent reaction between the label and the
15 substrate occurs and light energy is emitted. The liquid substrate, with the solid phase thus immersed in it, is exposed to the surface of the phosphor screen, and held so for a sufficient length of time to record or detect the emission pattern. This is preferably done through a transparent liquid-
20 retaining barrier, with the solid phase and the screen in sufficient proximity that the latter forms a well-defined image closely representative of the source array on the solid phase.

A convenient arrangement is one in which the solid phase is placed in a shallow enclosed receptacle filled with
25 the substrate solution such that the macromolecule pattern faces a transparent wall of the receptacle with a thin layer of the solution in between. The screen is held against the opposite side of the wall for the length of time referred to above. Minimum and optimal durations are matters of routine
30 experimentation which will be readily apparent to or determinable by those skilled in the art.

This invention has utility in a wide range of assays and laboratory procedures. Notable examples are protein assays, antibody assays, screening procedures, dilution
35 studies, DNA sequencing, and gene mapping. Other applications will be readily apparent to those skilled in the art.

Turning now to the drawings, FIG. 1 is a representation of one method of causing a chemiluminescent

reaction to occur in accordance with the invention and receiving the resulting emissions on a receptor material, and FIG. 2 is a diagram of an arrangement of components for stimulating the receptor material to emit signals corresponding to the emission it has received, and for sensing the signals and converting them to readable form.

FIG. 1 depicts a support 11 on which macromolecular species are immobilized. As indicated above, this may be a slab gel, a filter membrane, or a solid surface, depending on the type of procedure being conducted. The macromolecular species 12 itself is localized on the support surface in a distinct planar array, and has been tagged with the label 13. The entire support is placed in a transparent receptacle, such as a liquid-tight plastic bag 14, filled with the chemiluminescent substrate solution 15. As indicated in the general description above, the label is preferably an enzyme label, and the substrate solution preferably contains enough substrate to cause emissions to continue for at least 24 hours.

A phosphor screen 16 is placed over the plastic bag 15, directly above the immobilization pattern on the support, and held in this position for a sufficient period of time to receive sufficient emission to be detectable and yet show the same spatial arrangement as the immobilization pattern on the support. The phosphor in this illustration is one which traps the energy of the emission, and releases it only when stimulated with an external source such as infrared light.

Once the phosphors on the screen 16 are sufficiently excited, the screen is removed from contact with the plastic bag 14 and placed in the optical arrangement shown in FIG. 2. To permit a full two-dimensional scan of the screen, the screen is placed on a translating apparatus 21 which provides translation along the x- and y-axes. The x-stage translator component 22 and the y-stage translator component 23 of the apparatus are driven by an x-y translator controller 24.

The phosphor screen 16 is stimulated by light energy originating from an infrared laser 25 such as, for example, a Nd:YAG laser emitting light at 1064nm. The beam leaving the laser is collimated through collimating lenses 26 and deflected

by a YAG mirror 27. The beam is then focused on the phosphor screen 16 by a lens such as a 20× microscope objective 28 controlled by a z-axis micrometer 29. The translating apparatus 21 causes the beam to scan the entire surface of the screen.

Energy released from the phosphor screen by the infrared stimulation is deflected by a cold mirror 30 through a short pass filter 31 to a photomultiplier tube 32 powered by a high voltage power supply 33. The signal from the photomultiplier tube is directed to an oscilloscope 34 and a high speed digitizing voltmeter 35. The voltmeter reading is translated to a visual form by a graphics display 36 mediated by computer 37. The graphics display 36 permits a full reading and determination of the presence, location and amount of macromolecule immobilized on the support phase 11 of FIG. 1.

All components in this illustration may be supplied by conventional equipment and instrumentation well known and widely used in molecular biology laboratories. It is emphasized once again that this arrangement merely illustrates one method of practicing the invention. Others will readily come to mind to the routineer seeking to adapt the concept to a particular system, environment or available components.

The following example is offered for purposes of illustration. It is intended neither to define nor limit the invention in any manner.

EXAMPLE

A DNA strand was synthesized in a manner in which a limited number of UTP nucleotides with biotin molecules covalently attached were used in place of TTP nucleotides in a random primer labelling reaction. A dilution series of the DNA was then prepared, and a drop of each dilution was placed as a dot on a Zeta-Probe cationized nylon membrane (Bio-Rad Laboratories, Hercules, California). The resulting dots contained 100pg, 10pg, 1pg, 0.1pg and 0.01pg, respectively. The membrane was then incubated with a streptavidin-AP conjugate, with result that the DNA in all dots was labeled

with AP. The membrane was then placed in a plastic bag filled with an aqueous solution of 3-(2'-spiroadamantane)-4-methoxy-4-(3"-phosphoryloxy)phenyl-1,2-dioxetane (AMPPD), which upon undergoing chemiluminescence emits light at 470nm, at a concentration of 0.2mM and a total volume of 5mL.

An x-ray film (Kodak X-omat) was then placed against the plastic bag directly over the dots as shown in FIG. 1, and thus exposed to the AMPPD for ten minutes. The film was then developed by standard conventional x-ray film development procedures. Observation of the screen indicated that images corresponding to the dots representing 100pg, 10pg and 1pg were easily detected. An image of the 0.1pg dot could barely be detected, while no image of the 0.01pg dot could be detected.

The same plastic bag and contents were then placed against a Quantex Q-16 phosphor screen that had been prepared on an alumina substrate, in the same manner as the x-ray film, and likewise exposed for ten minutes. The screen is composed of a base of strontium sulfide, with samarium and cerium oxide as dopants, and barium sulfate and lithium fluoride as fluxes, with infrared sensitivity of 1120nm to 1220nm, a light emission curve peaking at 510nm, and is charged by light at 470nm. After the exposure, the screen was subjected to infrared laser scanning, using the apparatus depicted in FIG. 2. The results indicated that the phosphor screen was capable of clearly imaging the 100pg, 10pg, 1pg and 0.1pg dots, but not the 0.01pg dot.

The foregoing is offered primarily for purposes of illustration. It will be readily apparent to those skilled in the art that further variations, alternatives, substitutions and the like may be made without departing from the scope of the invention as claimed in the accompanying claims.

CLAIMS

1. A method for detecting macromolecules immobilized on a matrix, said method comprising:

5 (a) selectively tagging said macromolecules with a species capable of inducing a chemiluminescent reaction in a liquid-phase chemiluminescent substrate;

10 (b) placing said matrix, with said macromolecules so tagged, in contact with said liquid-phase chemiluminescent substrate to induce said chemiluminescent reaction;

(c) while said matrix and said liquid-phase chemiluminescent substrate are thus in contact, exposing said liquid-phase chemiluminescent substrate to a phosphor screen to excite phosphors thereon and thereby form an image corresponding to said macromolecules on said matrix; and

15 (d) detecting said image as an indication of the presence of said macromolecules on said matrix.

2. A method in accordance with claim 1 in which said liquid-phase chemiluminescent substrate emits, and said phosphors are excited by, light of at least one wavelength within the range of from about 400nm to about 600nm.

3. A method in accordance with claim 1 or claim 2 in which said macromolecules on said matrix comprise a plurality of distinct groups; step (a) comprises selectively tagging each said group with a distinct label; step (b) comprises placing
5 said matrix in contact with a liquid-phase mixture of a plurality of chemiluminescent substrates, each said substrate selectively inducible by one of said labels to undergo a chemiluminescent reaction emitting light at a wavelength distinct from that of other said substrates; and said phosphor
10 screen of step (c) contains a mixture of phosphors, each said phosphor selectively excitable by light at one of said wavelengths.

4. A method in accordance with any one of claims 1 to 3 in which said image of step (c) is a latent image, and step (d) comprises stimulating said phosphors by electromagnetic radiation consisting of visible light, x-rays, ultraviolet
5 radiation or infrared radiation to generate signals representative of said latent image.

5. A method in accordance with any one of claims 1 to 4 in which step (c) comprises exposing said liquid-phase chemiluminescent substrate to said phosphor screen through a transparent liquid-retaining barrier.

6. A method in accordance with any preceding claim in which said species of step (a) is conjugated to a binding member susceptible to an affinity-type binding interaction with said macromolecules, and step (a) comprises contacting said binding
5 member and said matrix to bind said binding member to any of said macromolecules present on said matrix.

7. A method in accordance with any preceding claim in which said species of step (a) is a phosphatase, said chemiluminescent reaction is an enzymatic reaction and said chemiluminescent substrate is one in which said
5 chemiluminescent reaction is rate enhanced by a phosphatase.

8. A method in accordance with any preceding claim in which said liquid-phase chemiluminescent substrate is an aqueous solution of a water-soluble compound capable of undergoing a chemiluminescent reaction.

9. A method in accordance with any preceding claim in which said matrix is a membrane with said macromolecules immobilised thereon in a planar array, in which said liquid-phase chemiluminescent substrate is retained in a receptacle having a
5 planar transparent wall, step (b) comprises immersing said membrane in said receptacle with said planar array of macromolecules adjacent to said planar transparent wall, said

phosphor screen of step (c) forms a planar solid surface, and
step (c) comprises holding said phosphor screen adjacent to
10 said planar transparent wall external to said receptacle.

10. A method in accordance with any preceding claim in
which step (b) comprises contacting said matrix with a sufficient
quantity of said liquid-phase chemiluminescent substrate to
cause chemiluminescent light emissions to continue for at least
5 about 24 hours.

11. A method substantially as hereinbefore described,
with particular reference to the drawings.